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Experimental evidence suggests that immunity to the Old World arenavirus, Lassa virus, is mediated by T cells. The related arenavirus, lymphocytic choriomeningitis virus (LCMV), cross-protects animals against lethal challenge with Lassa virus and animals cured of Lassa by ribavirin are protected against lethal challenge with LCMV. Thus, arenaviruses share protective antigenic determinants that are located on homologous regions of structural proteins. We tested a recombinant vaccinia virus which expresses the envelope glycoprotein precursor (GP-C) of Lassa virus (V-LSGP-C) for its ability to cross-protect mice against lethal challenge with LCMV, and have tried to locate T cell determinants which may

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19. Abstract (continued)

be involved in cross-protection. V-LSGP-C protects C3H/HEJ mice against challenge with LCMV, however, protection may not extend to all mouse strains. Mice primed with V-LSGP-C contain T cells which proliferate in response to LCMV *in vitro* and T cell lines which recognize both Lassa and LCMV GP-C have been established. Cross-reactive virus-specific CTL precursors could not be detected in the spleens of mice primed with V-LSGP-C. Eleven peptides were synthesized which correspond to segments of Lassa GP-C and which contain potential T cell determinants. All the peptides primed mice for a secondary peptide response *in vitro*, but only one of these peptides, corresponding with residues 403-417 of Lassa GP-C, induced specific proliferation of splenic lymphocytes from mice primed with V-LSGP-C or LCMV. This is the first molecular description of a T cell determinant which appears to be conserved between two species of Old World arenaviruses.

FOREWORD

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T CELL RESPONSES TO ARENAVIRUS INFECTIONS

INTRODUCTION

Arenaviruses are grouped serologically into two distinct categories (reviewed in 1) known as Old World and New World arenaviruses (Table 1). Some arenaviruses that are endemic in parts of Africa and Latin America cause severe hemorrhagic disease in susceptible human populations: in Africa, Lassa virus causes Lassa fever (2, 3), whereas in Argentina and Bolivia, Junin and Machupo viruses cause Argentinian hemorrhagic fever (reviewed in 4) and Bolivian hemorrhagic fever (5) respectively.

TABLE 1. Categories of selected arenaviruses

OLD WORLD ARENAVIRUSES	Location
Lymphocytic choriomeningitis	Worldwide
Lassa	West Africa
Mobala	Central African Republic
Mopeia	Mozambique
NEW WORLD ARENAVIRUSES	**Location**
Junin	Argentina
Machupo	Bolivia

Epidemiological evidence shows that Lassa virus places a heavy public health burden in endemic areas. There are an estimated 200,000 to 300,000 infections per year resulting in 3,000 to 5,000 deaths per year (6).

The mechanism of resistance to Lassa virus is poorly understood. There is very little evidence that antibodies play a role in recovery from Lassa fever even though antiviral antibodies can be detected by indirect fluorescence assays early after disease onset (reviewed in 7). Neutralizing antibodies (NT) appear late after the virus is cleared (7) and there is evidence that resistance to Lassa virus is mediated by virus-specific effector T cells. In a series of detailed experiments, Jahrling and Peters (7) found that inbred (strain 13) guinea pigs asymptomatically infected with the Armstrong strain of LCMV (LCMV-Arm) or with Mopeia virus were resistant to lethal challenge with Lassa virus. Moreover, guinea pigs cured of Lassa fever by ribavirin were resistant to challenge with the guinea pig-lethal WE strain of LCMV (7). Cross-protection could be transferred to normal recipients with splenic lymphocytes from immunized guinea pigs but not with their sera. Moreover, the splenic lymphocytes from such donors were cytolytic for arenavirus-infected target cells *in vitro* (7). Primates similarly immunized were also resistant to otherwise lethal challenge (8).

The cross-protection induced by Old World arenaviruses can be explained by the high degree of homology between the glycoproteins of Lassa and LCMV (9, 10). Therefore, at least some of the protective determinants recognized by effector T cells should reside within these regions of homology.

Recently, cDNA coding for Lassa glycoprotein precursor (GP-C) (11) and for nucleocapsid (N) (12) were inserted into vaccinia virus. These recombinants, each of which expresses the product of the gene-insert upon replication, are potential vaccine candidates and can serve as useful tools for assessing the relative contributions of N and GP-C proteins to the induction of virus-specific, cross-reactive immunity.

The long term goal of the work supported by the contract is to understand the nature of the immune response to Lassa virus with particular reference to the development of a specific vaccination strategy. Specific aims include the following:

1) Evaluate the immunogenicity and protective capacity of recombinant vaccinia viruses expressing the cDNA for structural proteins of Lassa.

2) Produce a series of murine T cell lines specific for structural proteins of LCMV and Lassa virus and characterize these cell lines as to phenotype, protein specificity and functional activity *in vitro* and *in vivo*.

3) Synthesize a series of peptides corresponding to potentially protective epitopes shared between, or unique to, LCMV and Lassa viruses and use them a) to define the fine specificity of T cells which might mediate protective immunity and b) as reagents which may have potential use in immunodiagnosis.

4). Use reactive peptides as potential probes for demonstrating and measuring virus-specific T cell responsiveness of monkeys which are immune to LCMV and Lassa virus.

MATERIALS AND METHODS

Mice

Mice were purchased from Jackson Laboratories (Bar Harbor, Maine) and used between 6 and 12 weeks of age. Virus-infected mice were housed in ventilated cage racks (Lab Products Inc, Aberdeen Md.). Unless stated otherwise, all experiments were conducted using C3H/HeJ mice.

Viruses

The "aggressive" strain of LCMV (UBC) was obtained from Dr. C. Pfau (Rensselaer Polytechnic Institute, Troy, NY). To prepare stock, the virus was passaged once in MDCK cells and then once in BHK cells at an MOI of 0.1. The culture fluid of 2-day-infected BHK cultures was used as a source of virus in those studies.

The Armstrong (Arm) strain of LCMV was obtained from Dr. P.B. Jarhling (USAMRIID, Ft Detrick, MD) and was passaged once in mouse brains. It was stored as a 10% (w/v) mouse brain suspension in PBS.

The recombinant vaccinia virus expressing the GP-C of Lassa virus (V-LSGP-C), the nucleoprotein (V-LSN) and the parental strain of vaccinia (Wyeth) were obtained from Drs. J. McCormick and D. Auperin (Special Pathogens Branch, CDC, Atlanta). Large stocks of these viruses were prepared in BSC-40 cells. Semiconfluent monolayers (70%) were infected with virus at MOI= 0.1 to 1. Cell-associated virus was obtained 2 days later by harvesting the cells and freeze-thawing them three times in a small volume of medium. All viruses were stored at -70° C.

Peptides

Peptides were synthesized "in house" using an Applied Biosystems 403A Peptide Synthesizer (Applied Biosystems, Foster City, CA) using the T-boc method.

Interleukin 2

Purified human interleukin 2 (IL-2) was obtained from Electro-Nucleonics Inc. (Silver Spring, MD) and used at a final concentration of 2%.

Immunization

Mice were immunized with LCMV (UBC) by intraperitoneal (ip) injection of of approximately 1000 PFU or virus in 0.2 ml PBS. The intracranial (ic) challenge dose of LCMV (UBC) was 200 PFU/mouse injected in a volume of 20 µl. For immunization with vaccinia or vaccinia constructs mice were primed with 10^6 PFU of virus ip unless otherwise stated.

For immunization with synthetic peptides mice were injected in the rear footpads with 100 µg of peptide in 50 µl of 50% complete Freund's adjuvant (CFA). Draining lymph node cells were excised 7 to 12 days later.

T cell proliferation assays

T cell proliferation assays were conducted in flat bottom 96 well tissue culture trays (Costar, Cambridge, MA). Cells were cultured in RPMI containing 10% fetal bovine serum (Hazelton, VA) glutamine, 10 mM HEPES buffer pH 7.5 and 5×10^{-5} 2 mercaptoethanol. Unless stated otherwise, lymph node cells or erythrocyte-depleted splenocytes were cultured at 4×10^5 viable cells per well for 4 days in 200 µl of medium with graded concentrations of peptides or viruses. To measure cell proliferation, cultures were pulsed with ^{3}H-thymidine (0.6 µCi/well; 20 mCi/mg specific activity) during the last four hours of the culture period. The cells were harvested using a PHD Cell Harvester (Cambridge Technologies, Cambridge, MA) and incorporated radioactivity was determined by liquid-scintillation counting in a Beckman LS1800 counter. Results were also expressed as a stimulation index (SI) which is defined as the ratio of the counts incorporated by cells cultured in the presence of peptide or virus to counts incorporated when the cells are cultured in medium alone.

Secondary Cytotoxic T cell induction and cytotoxicity assays

Anti-LCMV CTL were induced by culturing 1×10^7 erythrocyte-depleted splenic lymphocytes with 1×10^6 PFU of LCMV in 10 ml of medium. Five days later, the cultured cells were harvested, washed once, assessed for viability (>95%) and resuspended to an appropriate viable-cell concentration.

As a source of target cells, 6×10^5 L cells were infected with LCMV (UBC) at MOI=0.1 and cultured for 2 days in 75 cm^2 flasks containing 15 ml of medium. The target cells were trypsinized, washed once, and labelled with 100-200 mCi of $Na_2{}^{51}CrO_4$ for one hour at 37° C and then washed 5 times. Graded number of putative effector cells were cultured with $1\text{-}2 \times 10^4$ labelled target cells in 0.2 ml of medium in V-bottom wells for 6 hours at 37°. A 0.1 ml sample of medium from each of the wells was counted in a Beckman gamma counter. Cytolysis is defined as:

$$\%\text{cytolysis} = 100 \times \frac{(\text{experimental } {}^{51}\text{Cr release - spontaneous } {}^{51}\text{Cr release})}{\text{Total } {}^{51}\text{Cr - spontaneous } {}^{51}\text{Cr release}}$$

Virus-specific cytolysis is defined as: Cytolysis of virus-infected target cells minus the cytolysis of uninfected target cells.

Lytic units (LU) were derived as described by Nickell *et al* (13) with one LU being defined as the number of effector cells required to give 50 % virus-specific killing.

ELISA

Anti-peptide antibodies were measured in an ELISA using peptide absorbed to plastic. To coat the wells of 96 well ELISA trays with antigen, 50 µl of peptide solution in PBS was added to each of the wells and allowed to dry overnight. To block non-specific absorption of protein the dried wells were flooded with a 13.6% nonfat milk solution for 2 hours at room temperature. The blocking solution was replaced with 50 µl of test sera diluted in milk and incubated at room temperature for 1 hour. Unbound antibody was removed by washing the wells five times with TENT (50 mM Tris pH 7.5, 3mM EDTA, 500 mM NaCl and 0.05% Tween 20). Peroxidase-conjugated anti-mouse Ig was added to the wells in 50 µl of milk and incubated at room temperature for a further 1 hour. After washing the wells 5 times with TENT, the chromogenic substrate, o-phenylene-diamine, was added to the wells at 0.5 mg/ml in 0.1 ml of 0.1M citrate buffer (pH 4.5) containing H_2O_2 (5µl 30% H_2O_2 in 40 ml of buffer). After 30 minutes the reaction was stopped by the addition of 0.1 M NaF to all wells and absorbance was measured at 450 nm using a Dynatech ELISA reader.

Indirect immunofluorescence assays

Anti-LCMV antibodies were measured by indirect immunofluorescence (IFA) using spot slides of LCMV-infected and uninfected L cells. The air-dried cells were fixed with methanol for 3 minutes. Dilutions of antibody in PBS were added to the center of the cell spot in a volume of approximately 20 µl and incubated in a humidified box for 1 hour at 37°. After gently washing the slides in PBS, they were dried and 20 µl of FITC-conjugated goat anti-mouse antibody was added to the all spots and the slides incubated for a further 1 hour at 37°. The unbound second antibody was washed away and the slides counterstained by immersion in an aqueous solution (0.05% w/v) of Evans Blue. The slides were examined with a Zeiss fluorescence microscope.

Prediction of T Cell determinants

1). Detection of helical segments.

A computer program with the source code obtained from Margalit *et al* (14) was used to locate portions of the molecule showing an amphipathic helical secondary structure as proposed in the original contract application. The hydrophobicity scale of Hopp and Woods (15) was used for assigning hydrophobicity values to the amino acids in the sequence.

2). Detection of motifs.

The known amino acid sequences of known T cell determinants including those of viral origin contain amino acid motifs (16) consisting of a glycine or a charged amino acid followed by two or three hydrophobic residues and terminates with a glycine or a polar residue. We identified the presence of such motifs in the Lassa GP-C sequence using a word processing program (Microsoft Word) on a Macintosh microcomputer.

RESULTS

PROTECTION OF MICE AGAINST LCMV CHALLENGE BY IMMUNIZATION WITH VACCINIA-LASSA CONSTRUCTS

We tested the ability of the recombinant vaccinia construct, V-LSGP-C, to induce cross-protection against normally lethal ic challenge with LCMV. Table 2 summarizes the results from 5 experiments in which C3H/HeJ mice were primed with 10^6 PFU of V-LSGP-C and subsequently challenged at least one month later with LCMV. As shown 89% of mice primed with V-LSGP-C survived challenge compared with 12% of mice primed with parental vaccinia or with 5% of unprimed controls. Although the vaccinia-Lassa construct protected C3H/HeJ mice from death, immunity was not absolute because all mice surviving challenge showed transient symptoms of disease characteristic of lymphocytic choriomeningitis.

Table 2 Immunization with V-LSGP-C protects C3H/HeJ mice against LCMV challenge

IMMUNOGEN	SURV/TOTAL	%SURVIVORS
V-LSGP-C	24/27	89
VACCINIA	2/17	12
NONE	1/21	5
LCMV	10/10	100

C3H/HeJ mice were primed ip with 10^6 PFU of V-LSGP-C and vaccinia viruses and 10^3 PFU of LCMV then challenged i.c. at least 1 month later with $2x10^2$ PFU of LCMV (UBC). NONE vs V-LSGP-C, $p < 0.0005$; V-LSGP-C vs VACCINIA $p < 0.0005$; NONE vs VACCINIA $p > 0.05$; LCMV vs V-LSGP-C $p > 0.05$

The ability of V-LSGP-C to protect against LCMV may be genetically controlled in mice. In a single experiment, C57BL/6J mice primed with a ten-fold higher dose of V-LSGP-C (10^7 PFU) succumbed to i.c. challenge with LCMV (Table 3).

Table 3 C57BL/6J mice are not protected against LCMV challenge by immunization with V-LSGP-C

IMMUNIZATION	SURVIVORS/TOTAL
LCMV	5/5
VLS	1/5
VAX	1/5
NORMAL	0/6

MECHANISM OF CROSS-PROTECTION INDUCED BY V-LSGP-C

Work from this and other laboratories have established that immunity to LCMV infection, and the immunopathology of lymphocytic choriomeningitis, are mediated by virus-specific cytotoxic T cells (17, 18). Therefore, we presumed that V-LSGP-C mediated cross-protection against LCMV challenge by inducing CTL specific for antigenic determinants common to both LCMV and Lassa virus GP-C. We therefore looked for such cross-reactive T cells using lymphoproliferative assays and cytotoxicity assays.

V-LSGP-C primes for cross-reactive T cells

Table 4 shows the results of 3 experiments in which splenic lymphocytes from V-LSGP-C-primed mice were cultured with LCMV *in vitro.* In experiment 1 their response to LCMV was equivocal and markedly lower than that of splenic lymphocytes from LCMV-primed mice. However, in the remaining experiments V-LSGPC-primed splenic lymphocytes made stronger responses to LCMV. These results show that the construct does prime mice for against LCMV but that the degree of priming probably varies from experiment to experiment.

Table 4 V-LSGP-C induces cross-reactive T cells

	IMMUNOGEN			
LCMV (PFU/well)	V-LSGP-C	VACC	LCMV	NONE
Experiment 1				
0	1600	1963	1021	5700
$1x10^4$	900	1909	34000	6264
$3x10^4$	700	1248	32000	6273
$1x10^5$	3250	1260	33000	8142
Experiment 2				
0	1641	ND	ND	496
$1x10^4$	4854	ND	ND	983
$3x10^4$	8687	ND	ND	1244
$1x10^5$	13247	ND	ND	980
Experiment 3				
0	819	ND	ND	982
$1x10^4$	19307	ND	ND	928
$3x10^4$	34012	ND	ND	1370
$1x10^5$	45539	ND	ND	1471

Splenic lymphocytes from normal or virus-primed mice were cultured at $4x10^5$ cells per well for 4 days with LCMV. Proliferation was measured by ^{3}H-Thymidine incorporation. ND = not done.

Failure to detect cross-reactive CTL precursors in V-LSGP-C-primed mice

Splenic lymphocytes from V-LSGP-C -primed mice were cultured with LCMV for 5 days. Cytotoxic activity of the cultured cells against LCMV-infected L cells was measured in a ^{51}Cr release assay. The results of a typical experiment is shown in Table 5. We consistently found that LCMV-specific lytic activity could not be detected in the spleens of mice primed with V-LSGP-C. On the other hand, cells from LCMV-primed mice exhibited high levels of virus-specific cytotoxic activity under the same experimental conditions. It appears that, at the dose used, V-LSGP-C either does not prime for cross-reactive CTL or, less likely, that the frequency of such cells may be below the limit of detection.

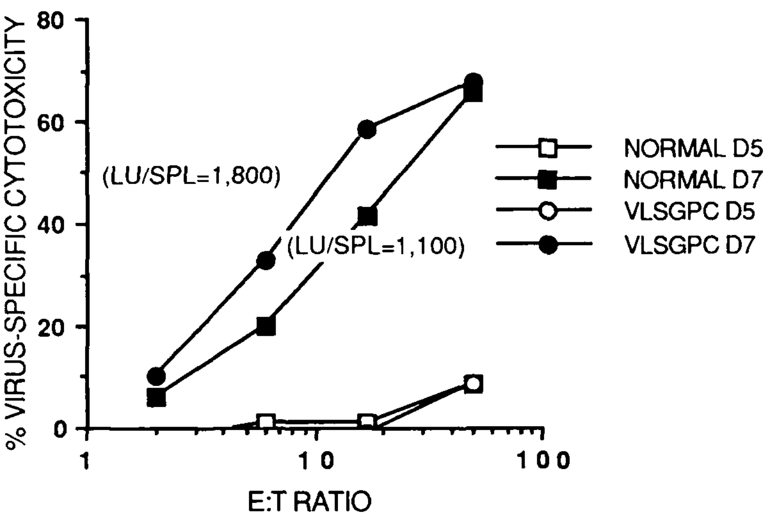
Even though cross-reactive CTL precursors seemed to be absent in the spleens of mice primed once with V-LSGP-C, we explored the possibility that these mice could exhibit an accelerated rate of CTL induction or greater CTL activity after LCMV challenge. Therefore, we compared the rates of CTL induction in the spleens of construct-primed and normal mice after i.p. challenge with LCMV. Fig. 1 shows that virus-specific CTL were not detectable at day 5 in either group. At day 7, both groups of mice showed high CTL activity with the splenic lymphocytes from construct-primed mice showing, at most, a two fold higher level of lytic units (LU) per spleen. This result does not support the notion that protection induced by V-LSGP-C immunization is due to a more rapid induction of CTL after LCMV challenge.

Table 5 V-LSGP-C does not induce detectable cross-reactive CTL

	%VIRUS-SPECIFIC CYTOTOXICITY @ E:T			
IMMUNOGEN:	50	17	6	2
LCMV	79.8	72.3	45.6	23.1
V-LSGP-C	0	0	0	0
VACCINIA	0	0	0	0
NONE	0	0	0	0

Splenic lymphocytes from normal and virus-primed mice were cultured ($1.5x10^7$/flask) with 10^6 PFU of LCMV. On day 4 the viable cells were harvested and tested for LCMV-specific CTL in a ^{51}Cr release assay using LCMV-infected L cells. Cytolysis of < 5% was designated as 0% for the purposes of clarity.

Fig. 1 Prior priming of mice with V-LSGP-C does not alter course of CTL induction in response to LCMV challenge.



Groups of normal C3H/HeJ mice and those primed 28 days previously with 10^6PFU V-LSGP-C were challenged with 200 PFU LCMV (UBC) five or seven days prior to the CTL assay. On the same day two mice per group were killed and pooled spleen cell suspensions were tested for LCMV specific CTL in a ^{51}Cr release assay employing LCMV-infected L cells as targets. One lytic unit (LU) is defined as the number of splenic lymphocytes required to give 50% virus-specific lysis.

Are cross-reactive arenavirus-specific antibodies relevant to protection?

The possibility that cross-protection is mediated by cross-reactive antibodies is unlikely. C3H/HeJ mice required at least two doses of 10^7 PFU of V-LSGP-C in order to develop anti-Lassa antibodies that were detectable by indirect immunofluorescence analysis on Lassa virus-infected cells. However, these sera contained no detectable antibodies against LCMV-infected cells. No Lassa-specific antibodies were detectable after a single dose of 10^7 PFU of V-LSGP-C. These data suggest that antibodies are not involved in cross-protection.

Production of cross-reactive T cell lines specific for GP-C determinants shared by both Lassa virus and LCMV

As part of our study of the mechanism of protection by V-LSGP-C, we have established T cell lines whose specificity appears to be directed against determinants common to both Lassa virus and LCMV GP-C. These lines were derived from spleens of C3H/HeJ mice which had been primed with LCMV ip and subsequently challenged i.c. with the same virus. Splenic lymphocytes from these mice were cultured with nonimmune syngeneic splenocytes infected *in vitro* with V-LSGP-C. Thereafter, the resulting blast cells were restimulated on a weekly basis with V-LSGP-C-infected splenocytes. After the second such stimulation, exogenous IL-2 (18) was included in the culture medium. To test the specificity of these lines, the T cells were allowed to revert to small "resting" lymphocytes by culturing them with syngeneic splenocytes in the absence of virus and IL-2. The rested cells were restimulated (in the presence of IL-2 and syngeneic splenic lymphocytes) with either V-LSGP-C, LCMV or VLSN. The results in Table 6 show that both T cell lines responded well to V-LSGP-C and LCMV but not VLSN construct suggesting that they are specific for determinants on the GP-C of Lassa virus and LCMV. The functional activities of these cells and their derived clones will be studied *in vitro* and *in vivo* .

Table 6 Specificity of cross-reactive T cell lines

	Line 1		Line 2	
Stimulus (*in vitro*)	^{3}H-TdR incorp (cpm ± sem)	Stimulation Index	^{3}H-TdR incorp (cpm ± sem)	Stimulation Index
V-LSGP-C	8386 ± 448	7.7	8797 ± 507	7.5
LCMV (UBC)	8534 ± 384	7.8	9204 ± 511	7.9
VLSN	2728 ± 534	2.5	2894 ± 577	2.5
Uninfected splenic lymphocytes	1086 ± 73		1167 ± 74	

T cell blasts were cultures with uninfected syngeneic feeder cells in the absence of antigen or IL2 to allow them to revert to small resting lymphocytes. After 7 days they were harvested and tested in a T cell proliferation assay. 10^4 T cells were cultured with 5 x10^5 normal splenic lymphocytes or 5 x10^5 splenic lymphocytes infected (MOI=0.1) with V-LSGP-C, LCMV or V-LSN and 2% IL-2.

MAPPING OF T CELL DETERMINANTS ON LASSA VIRUS GP-C

In recent years the physiochemical characterization of T cell determinants has been a subject of great interest. T cells generally recognize linear fragments of processed antigen presented to them in association with Class I or Class II antigens of the Major Histocompatibility Complex on stimulator cells (reviewed in 19). In contrast, B cells recognize determinants which may consist of either linear sequences or those created when amino acids from distal parts of the protein are brought together during folding of the molecule into its tertiary structure.

Studies of known T cell determinants suggest that they possess an amphipathic helical secondary structure (14) and/or that they have a characteristic motif of amino acids with particular physicochemical properties (16). Predictive schemes (algorithms) have been devised which locate these structures on protein molecules (14, 16) and we have used them (as described in detail in Materials and Methods) to localize potential T cell determinants on the Lassa virus GP-C. The amino acid sequence of Lassa GP-C is shown in Table 7; the regions showing an amphipathic helical structure are shown in bold type and the underlined segments contain the relevant amino acid motifs.

Table 7 Potential T Cell determinants on Lassa virus glycoprotein (GP-C)

BOLD TYPE: SECTIONS PREDICTED TO HAVE AN AMPHIPATHIC HELICAL STRUCTURE.
UNDERSCORED: MOTIFS.

MGQIVTFFQE	**VPHVIEEVMN**	**IVLIALSVLA**	VLKGLYNFAT	CGLVGLVT**FL**	50
LLCGRSCTTS	**LYK**GVYELQT	LEL**NMETLNM**	**TMPLSCTKNN**	**SHHYI**MVGNE	100
TGLEL**TLTNT**	**SIINHKFCNL**	SDAHKKNLYD	HALMSIISTF	HLSIPNFNQY	150
EAMSCDFNGG	KISVQYNLS**H**	**SYAG**DAANHC	GTVANGVLQT	FMRMAWGGSY	200
IALDS**GRGNW**	**DCIMTSYQYL**	IIQNTTWEDH	CQFSRPSPIG	YLGLLSQRTR	250
DIYISRRLLG	TFTWTLSDSE	GKDTPGGYCL	TRWMLIEAEL	KCFGNTAVAK	300
CNEKHDEEFC	DMLRL**FDFNK**	**QAIQRL**KAE**A**	**QMS**IQLINKA	VNAL**INDQLI**	350
MKNH**LRDIMG**	**IPYC**NYSKYW	YLNH**TTTGRT**	SLPKCWLVSN	GSYLNETHFS	400
DDIEQQADN**M**	**ITEMLQKEYM**	ERQGKTPLGL	VDLFVFSTSF	YLISIFL**HLV**	450
KIPTHRHIVG	KSCPKPHRLN	HMGICSCGLY	KQPGVPVKWK	R	500

We synthesized 11 peptides, arbitrarily designated Series 1 and Series 2 peptides, which contained potential T cell determinants from Lassa virus GP-C. Series 1 peptides were chosen because they were predicted by one or both algorithms to contain T cell determinants; these peptides included regions of GP-C showing either high or low homology between LCMC and Lassa virus. Series 2 peptides which are overlapping 15mers, were selected (independently of any algorithm) simply because they are from regions of Lassa and LCMV GP-C which are highly homologous and thus were predicted to contain protective determinants. These regions include residues 381-417 (89% homology) and residues 436-470 (86% homology) of Lassa GP-C. Four of the 6 Series 2 peptides have the motifs and/or amphipathic helical structure of predicted T cell determinants. The peptide sequences, their positions on the Lassa GP-C molecule, and the corresponding sequences from LCMV (Arm) GP-C are shown in Table 8.

We characterized these peptides for their respective abilities to prime T cells from C3H/HeJ (H-2^k) mice for a proliferative response *in vitro*. Our findings, summarized in Fig. 2 and Fig. 3, show that each peptide induced a secondary reponses of varying magnitude. None of these peptides induced T cell proliferation in lymphoid cells from normal unprimed mice.

In addition to the response of C3H/HeJ (H-2^k) mice, we looked at the ability of other mouse strains, C57BL/6J (H-2^b), BALB/c (H-2^d) and DBA/1 (H-2^q) to respond to Series 1 peptides. Each peptide was tested sequentially in each of 4 strains of mice.

(Experiment 4) was immunogenic in mice of all four strains, and in this experiment the highest responses were mounted by BALB/c (H-2^d) and DBA/1 (H-2^q) mice. In experiment 2, peptide GP-C 170-183 induced a

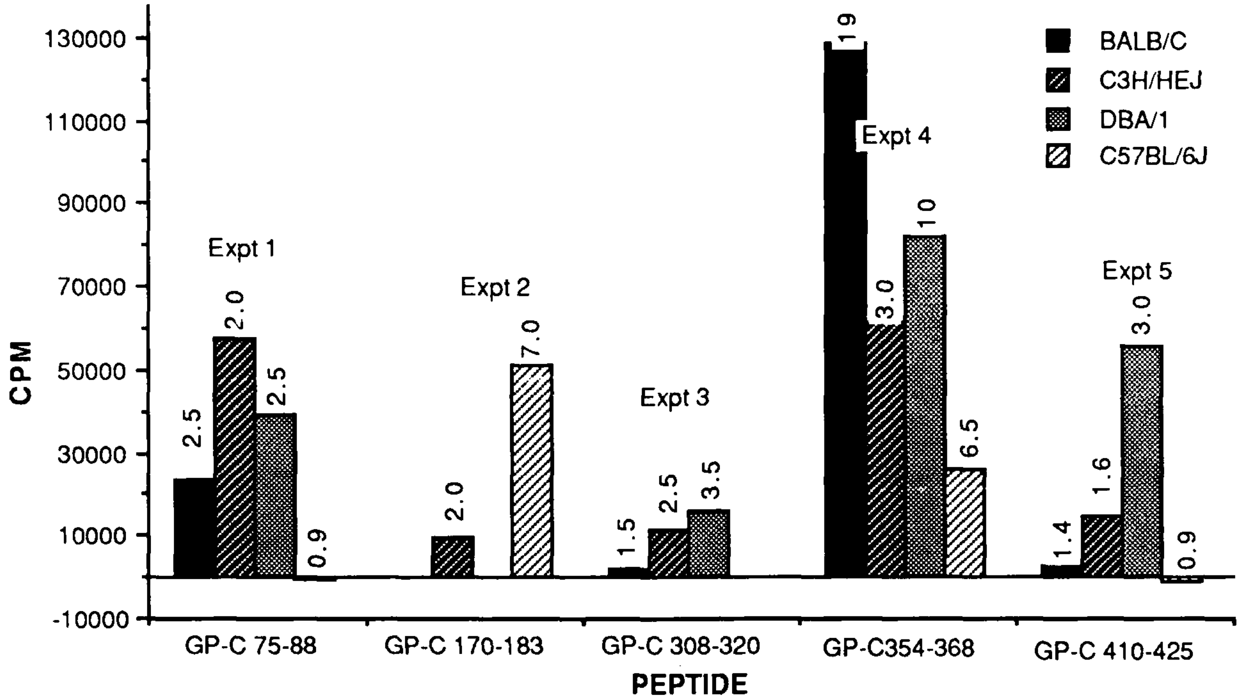
Table 8 Synthetic peptides corresponding to potential T cell determinants on Lassa GP-C

PEPTIDE DESIGNATION*	SEQUENCE	MOTIF	HELIX
SERIES 1			
GP-C 75-88.	LASSA **METLNMTMPLSCT** (LCMV MSHLNLTMPNACS)	NO	YES
GP-C 170-183.	LASSA **HSYAGDAANHCG** (LCMV FSDAQSAQSQCR)	YES	YES
GP-C 308-320.	LASSA **EFCDMLRLFDFNK** (LCMV EFCDMLRLIDYNK)	YES	YES
GP-C 354-368.	LASSA **HLRDIMGIPYCNYSK** (LCMV HLRDLMGVPYCNYSK)	YES	YES
GP-C 410-425.	LASSA **MITEMLQKEYMERQGK** (LCMV MITEMLRKDYIKRQGS)	YES	YES
SERIES 2			
GP-C 383-397.	LASSA **PKCWLVSNGSYLNET** (LCMV PKCWLVTNGSYLNET)	NO	NO
GP-C 393-407.	LASSA **YLNETHFSDDIEQQA** (LCMV YLNETHFSDQIEQEA)	NO	NO
GP-C 403-417.	LASSA **IEQQADNMITEMLQK** (LCMV IEQEADNMITEMLRK)	YES	YES
GP-C 436-450.	LASSA **FSTSFYLISIFLHLV** (LCMV FSTSAYLVSIFLHLV)	YES	YES
GP-C 446-460.	LASSA **FLHLVKIPTHRHIVG** (LCMV FLHLVKIPTHRHIKG)	YES	YES
GP-C 456-470.	LASSA **RHIVGKSCPKPHRLN** (LCMV RHIKGGSCPKPHRLT)	YES	NO

* The numbers in the peptide designation refer to the boundaries of the peptide residues on the GP-C molecule

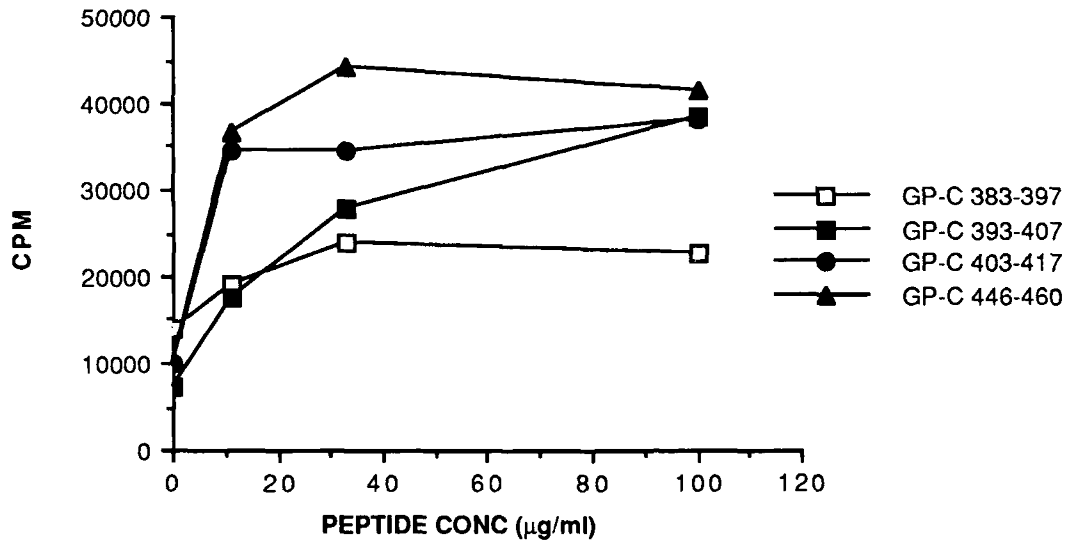
moderate response in C57BL/6J mice. The other peptides were poorly immunogenic because they induced responses which were, at most, 2 to 3-fold above background. None of the Series 1 peptides appeared to be virus-specific because none of them consistently induced lymphoproliferation of V-LSGP-C or LCMV-primed splenic lymphocytes nor did these viruses stimulate peptide-primed lymphocytes (data not shown).

Fig. 2 Priming of mice by Series 1 peptides for a secondary proliferative response *in vitro*



Mice were primed in the footpads with 100 µg of peptide in CFA and 7-12 days later their draining lymph node cells were tested in a proliferation assay. Each peptide was tested in a separate experiment in which the responses of the various mouse strains was compared. The peptides were titrated in the assay from 100 µg/ml to 1 µg/ml and the resulting proliferation was dependent on peptide concentration. As the response plateaued between 100 and 33 µg/ml, only the results for 33 µg/ml are presented. The stimulation indices are shown above the bars.

Fig. 3 Series 2 peptides prime C3H/HeJ mice for secondary lymphoproliferative responses



C3H/HeJ mice were primed in the footpad with 100 µg of peptide emulsified in CFA. Their draining lymph node cells were used in a proliferation assay 8 days later.

Series 2 peptide defines a T cell determinant conserved on GP-C from Lassa virus and LCMV.

To determine if lymphocytes from V-LSGPC-primed mice which survived a normally lethal LCMV challenge were primed to determinants in the highly conserved regions of GP-C, splenic lymphocytes from such mice were tested for responsiveness to Series 2 peptides. Table 9 shows that lymphocytes from such mice indeed proliferated in response to GP-C 403-417.

Table 9 Virus-specific responsiveness to a Series 2 peptide

PEPTIDE	PROLIFERATION CPM (SI) LCMV SURVIVORS MOUSE 1		MOUSE 2		NORMAL	
GP-C 383-397	589	(0.3)	857	(0.3)	234	(0.4)
GP-C 393-407	1019	(0.5)	1755	(0.3)	623	(1)
GP-C 403-417	**9390**	(5)	**18386**	(7)	567	(1)
GP-C 446-460	1123	(0.6)	2137	(0.8)	540	(1)
GP-C 456-470	876	(0.4)	1196	(0.4)	508	(1)
LCMV	5466	(3)	9472	(3.5)	328	(0.6)
CONTROL	1954		2715		558	

C3H/HeJ mice were primed with 10^7 PFU of V-LSGP-C ip and challenged 36 days later with 10 i.c. LD_{50} of LCMV (ARM). Their splenic lymphocytes were tested for reactivity to peptides 27 days later. The concentration of peptides used was 50 μg/ml.

The results presented in Table 10 show that splenic lymphocytes from mice primed with either V-LSGP-C or LCMV are also responsive to GP-C 403-417, however, V-LSGP-C-primed mice made a much lower response than their LCMV-primed counterparts. This suggests that, in the context of GP-C, the construct is much less immunogenic than LCMV. Normal mice or mice primed to Lassa nucleocapsid with the VLSN construct did not respond to this peptide. Thus, GP-C 403-417 appears to define a virus-specific T cell determinant common to GP-C from Lassa virus and LCMV.

Anti-peptide antibodies

As part of our characterization of Series 1 peptides, we wished to determine whether priming with these peptides would stimulate a specific antibody response. The sera of mice which had received two injections of GP-C 354-368 were tested in an ELISA. As shown in Fig. 4, sera from C57BL/6J and DBA/1 mice contained antibodies to the peptide but C3H/HeJ mice, which were capable of mounting a T cell response to it, made no such antibodies. Sera from control mice (primed only with CFA) contained no such antibodies. The specificity of the anti-peptide response in C57BL/6J mice to GP-C354-358 was confirmed in a competitive binding (peptide inhibition assay) (Fig. 5). Antibody activity was inhibited by GP-C 354-368 but not by GP-C 308-320. Sera from mice infected with LCMV or V-LSGP-C were screened for antibodies to GP-C 354-368, but none were found suggesting that the B cell determinant on GP-C 354-368 is not virus-specific .

We also tested the sera from mice primed with the other Series 1 peptides, 75-88, 170-183, and 410-425, for specific antibodies. Fig. 6 shows that GP-C 170-183 induced significant levels of anti-peptide antibodies in C3H/HeJ mice but not in C57BL/6J mice. However, in both these mouse strains, T cell responses to GP-C 170-183 were demonstrable. This indicates that presumably peptides recognized by T cells do not necessarily stimulate antibody responses even though they prime for a secondary lymphoproliferative response.

Table 10 The response of lymphocytes from mice primed with LCMV or V-LSGP-C to the Series 2 peptide, GP-C 403-417

PROLIFERATION
CPM (Stimulation Index)

Experiment 1

Peptide conc (μg/ml)	VLSGPC		VLSN		LCMV (UBC)	
30	2341	(3.9)	2039	(0.8)	9371	(19.5)
10	2245	(3.8)	2797	(1.1)	11039	(23.0)
3	2200	(3.7)	2749	(1.1)	9477	(19.7)
1	1775	(3.0)	2645	(1.0)	7989	(16.6)
0 (control)	590		2536		480	

Experiment 2

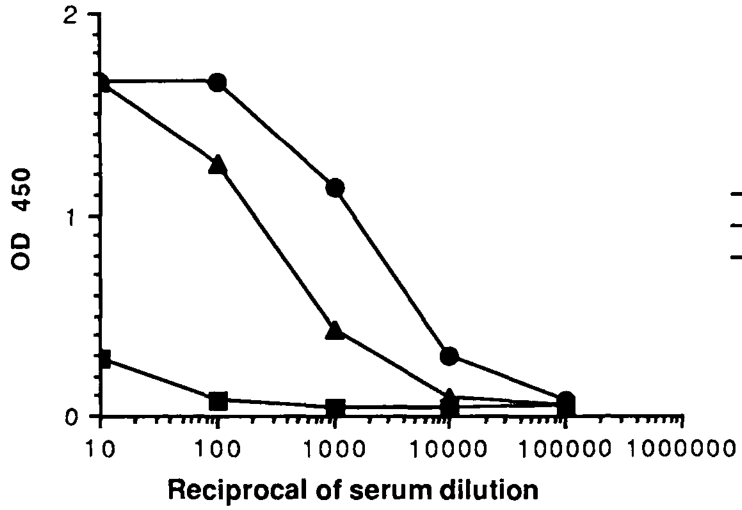
Peptide conc (μg/ml)	VLSGPC ip† LCMV(Arm) ic		LCMV (ARM)		LCMV (UBC)		NORMAL	
50	12251	(9)	11591	(21)	15378	(3)	837	(1)
16.7	16941	(13)	19134	(35)	17654	(4)	1099	(1)
5.5	14239	(11)	15361	(28)	16847	(3)	759	(1)
0 (control)	1347		554		4949		918	

Experiment 1. Mice were primed with either 10^7 PFU of VLSGPC or VLSN 30 days prior, or 500 PFU of LCMV (UBC) 200 days previously.

Experiment 2. †Mice were primed with 10^7 PFU of V-LSGP-C ip and challenged 36 days later with 10 i.c. LD_{50} of LCMV (ARM). They were used 31 days after challenge.

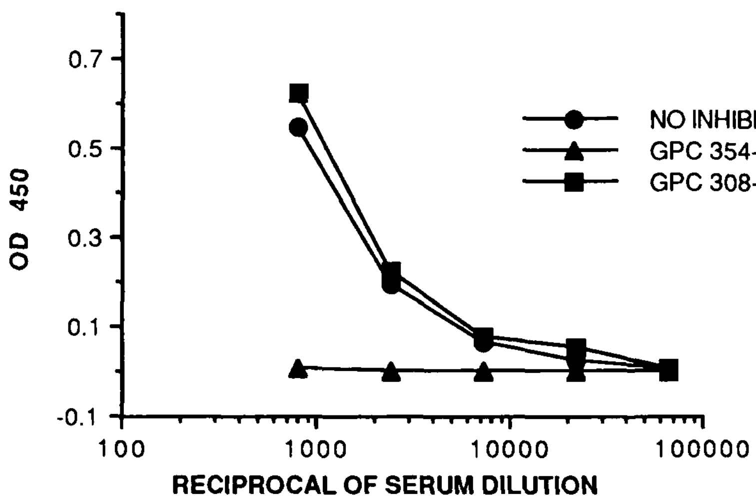
LCMV (Arm) and LCMV (UBC) primed mice were used 34 and 209 days respectively after ip priming

<u>Fig. 4 Anti-peptide antibodies in sera of mice primed with the Series 1 peptide, GP-C 354-368</u>



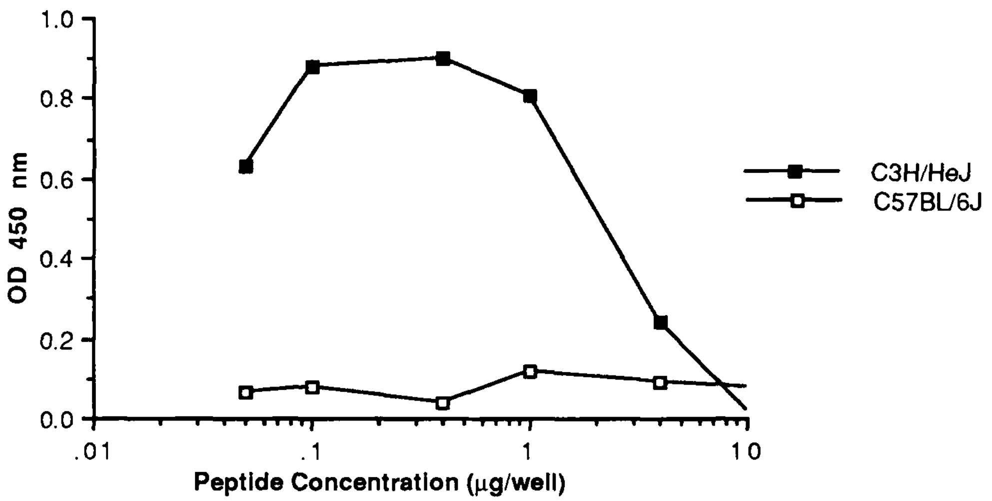
Mice were primed with 100µg GP-C 354-368 in CFA and boosted 3 months (footpad) and 5 months later (ip) with 100µg of peptide in incomplete adjuvant. They were bled 13 days after the last injection and serum dilutions were tested in an ELISA against wells coated with 0.57 µg/well of GP-C 354-368.

<u>FIG 5 Peptide inhibition of antibodies to Series 1 peptide, GP-C 354-368</u>



Dilutions of antisera were incubated overnight with 200 nmole/ml of GP-C 354-368 or GP-C 308-320 and then tested in an ELISA assay against GP-C 354-368

Fig. 6 Antibody response to the Series 1 peptide, GP-C 170-183



Mice were primed with 100µg of GP-C 170-183 in CFA and boosted 3 months (footpad) and 5 months later (ip) with 100µg of peptide in incomplete adjuvant. They were bled 13 days after the last injection and a 1/100 dilution of serum was tested in an ELISA against wells coated with titrated amounts of peptide.

CONCLUSIONS

This report summarizes our progress in a) assessing the capacity of a recombinant vaccinia-Lassa construct, V-LSGP-C to induce protective immunity in mice against fatal LCMV challenge and b) delineating possible T cell epitopes on the Lassa virus GP-C which may contribute to protection.

Immunization of C3H/HeJ mice with 10^6 PFU of V-LSGP-C protects them against subsequent normally lethal i.c. challenge with LCMV. This extends previous findings that V-LSGP-C protects guinea pigs and primates against Lassa virus (11,20) and suggests that an immune response against GP-C may be sufficient for cross-protection. However, it appears that this response may not be sufficient to prevent the transient appearance of disease after challenge with LCMV. Incomplete protection has also been seen when V-LSGP-C-primed guinea pigs and primates were challenged with Lassa virus (11,20). Since a dose of 10^6 PFU of the construct may be sub-optimal for inducing protection, these experiments are being repeated using a 10-fold higher doses of virus. Shortly, we expect to receive from Dr David Auperin (CDC, Atlanta) a new construct in which the GP-C gene is inserted into the more replication-competent WR strain of vaccinia. Constructs made using this strain are likely to be more immunogenic because they are able to persist longer after inoculation than those made using the Wyeth strain (21) and, therefore, would effectively deliver a larger dose of GP-C.

Despite the impressive results in C3H/HeJ mice, C57Bl/6J mice were not protected against LCMV challenge when primed with a ten-fold higher dose of V-LSGP-C (10^7 PFU/mouse). Reasons for the strain differences are not clear at this time.

The mechanism of cross-protection induced by V-LSGP-C in C3H/HeJ mice is under investigation. It appears that antibodies play no part in cross-protection because no anti-LCMV antibodies were found by immunofluorescence in the sera of C3H/HeJ mice primed with up to two doses of 10^7 PFU of V-LSGP-C. This is consistent with the finding that primates primed with V-LSGP-C had a low titer of only non-neutralizing antibodies to Lassa GP-1 and GP-2 prior to challenge and that NT Abs did not appear in these primates until at least 21 days after Lassa challenge and in some monkeys not until day 98 (20). Monkeys cross-protected by immunization with Mopeia virus did not develop NT Abs to Lassa virus at any time before or after challenge (20).

As predicted, V-LSGP-C-primed T cells were cross-reactive with LCMV, however, the degree of reactivity varied between experiments. We have not yet been able to detect LCMV-specific CTL precursors in the spleens of such mice. Given the possibility that the frequency of such precursors may be too low to detect in mice primed with 10^6 PFU of construct, we will be repeating these experiments with greater (and multiple) doses of V-LSGP-C, and, eventually we will use the new construct mentioned above to prime mice. In addition, the development of cross-reactive T cell lines and clones, such as the two described in this report, and the analysis of their functional and phenotypic properties, will help delineate the protective mechanisms at play in mice primed with V-LSGP-C.

The two algorithms used in our studies suggest that there are at least 25 potential T cell determinants on Lassa virus GP-C. Series 1 peptides represent 5 such determinants and two of these, GP-C 354-368 and GP-C 170-183 were found to be immunogenic for mice when given in complete Freund's adjuvant. They also induced an antibody response when given to the appropriate mouse strain (GP-C 354-368 in C57BL/6J and DBA/1 mice and GP-C 170-183 in C3H/HeJ mice) which indicates that they are capable of inducing T helper cells. The finding that, in some mouse strains a peptide will not induce antibodies despite its capacity to prime T cells, suggests that different T cell subsets may be primed in these mouse strains (22). Despite our finding that two of the Series 1 peptides were immunogenic, none were found to stimulate V-LSGPC or LCMV-primed splenic lymphocytes suggesting that they are not virus-specific.

Series 2 peptides may be more promising for defining protective T cell epitopes on Lassa GP-C. The synthetic peptide, GP-C 403-417, contains a virus-specific T cell determinant which consistently induced proliferation of T cells from LCMV-primed mice, and, to a lesser extent, T cells primed with V-LSGP-C. Again, the reduced response of V-LSGPC-primed splenic lymphocytes to this peptide most likely reflects a lesser degree of priming by the vaccinia-construct. The lack of stimulation by the overlapping peptide, GP-C 393-407, suggests that the relevantT cell determinant is not associated with the 5 residue stretch shared with GP-C 403-407 (IEQQA) and that it is more likely to be located in carboxyl terminal region of the peptide which contains an amphipathic helix and amino acid motifs (see Tables 7 and 8).

The limitation of using the current algorithms for locating T cell determinants is borne out by the finding that only 1 out of 9 Lassa GP-C peptides predicted to contain a T cell determinant was virus-specific. In a recent report, only one of 16 potential T cell determinants on the influenza matrix protein was recognized by matrix protein-specific CTL clones (23) which points to a role of other factors in determining whether or not a given T cell determinant is "selected" by the immune system (24).

In summary, we have demonstrated that cross-protection to arenaviruses can be mediated by an immune response to Lassa viral envelope glycoproteins expressed by recombinant vaccinia virus. A virus-specific T cell determinant on GP-C has been defined which appears to be common to Lassa virus and LCMV. Our focus for the remainder of the year will be to establish the relevance of the conserved T cell determinant (and others that we may find) to virus-specific protection.

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